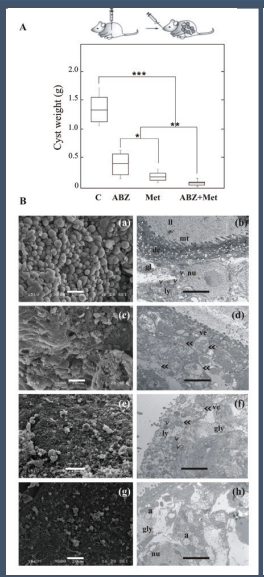
Role of FoxO in the regulation of Metformin-stimulated energy stress in *Echinococcus* spp.

Julia A Loos,[1,2] Valeria A Dávila[1,2], Andrea C Cumino.[1,2]
[1] CONICET, [2] Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Mar del Plata

ABSTRACT

In a wide range of organisms the Forkhead transcription factor FoxO and the SIRT deacetylase constitute a nutrient-sensing pathway involved in regulation of multiple cellular functions. FoxO proteins function mainly as transcriptional activators by binding the consensus core recognition motif TTGTTTAC, and their activity is inhibited by the insulin and IGF-1 signaling pathway. Conversely, in the absence of growth factor signaling or upon cellular stress, FoxOs translocate into the nucleus and activate FoxO-dependent gene expression. Currently, this pathway remains unknown in *Echinococcus* spp. We have previously shown that Metformin (Met), an anti-hyperglycemic and anti-proliferative drug, exhibits considerable *in vitro* and *in vivo* activity against *E. granulosus* metacestodes. Here, we extended the study and demonstrated that the drug also possess chemopreventive properties against alveolar echinococcosis in mice. As drug administration was shown to induce the Eg-AMPK activation, its anti-echinococcal effects might be a consequence of cellular energy charge depletion in the parasite. Based on this and the fact that only one FoxO transcription factor is present in the genome of *Echinococcus* spp, the aim of this work is investigate the activation state of FoxO and its relation with the expression of genes encoding key autophagy-related proteins in parasites incubated under both control and energy-stress conditions. Eg-FoxO sequence reveals several post-translationally modifiable residues highly conserved. By *in toto* immunolocalization assays, we detected the expression and subcellular localization of a phosphorylated (Ser352) and an acetylated (Lys373) form of Eg-FoxO in control and Met-treated protoscoleces. Interestingly, similar expression patterns were observed in both samples. Additionally, by qPCR analysis, we found that Met produced an increase in the transcriptional expression *atg* genes in E. *granulosus* protoscoleces and metacestodes and in *E. multilocularis* primary cells. In this regards, BLASTn analysis of the upstream sequences in putative promoters of several of these genes showed the conserved binding motif described for FoxO-activated genes. These results suggest a possible role of FoxO in the transcriptional regulation of *Echinococcus* spp. under energy stress conditions. We also detected expression of Atg8 polypeptide (LC3) with both a diffuse and punctate staining in control and Met-treated *E. granulosus* protoscoleces and *E. multilocularis* vesicles. However, western blot analysis demonstrated higher levels of Eg-Atg8-PE (LC3-II) in Met-treated protoscoleces, suggesting a possible induction of autophagy under this condition. Altogether, our data indicate that FoxO and autophagy might participate in the regulation of Met-stimulated energy stress in *Echinococcus* spp.

RESULTS



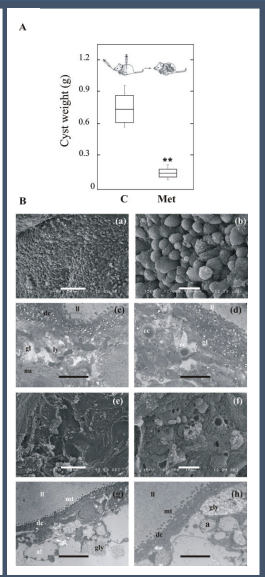


Fig 1. Therapeutic efficacy study in *E. granulosus* infected mice. (A) Box plots showing the comparative distribution of the weight (g) of cysts recovered from untreated mice (C) and treated with albendazole (ABZ, 5 mg/kg/d), metformin (Met, 50 mg/kg/d) and the combination of both drugs (ABZ+Met) for 60 days after 4 months of infection. The weight of cysts was significantly decreased upon all treatments compared with the control (***$p < 0.01$), but the decrease was more prominent in the group receiving the combined treatment than in those with either drug alone (**$p < 0.05$); in turn, weight reduction was greater with Met than with ABZ (*$p < 0.2$). (B) Representative SEM (a, c, e, g) and TEM (b, d, f, h) images of hydatid cysts recovered from untreated mice (a, b) or treated with Met (c, d), ABZ (e, f) and ABZ+Met (g, h). Bars indicate: 20 µm in (a, c, e, g); 1 µm in (b, d, f, h).

Fig 2. Chemoprophylactic activity of metformin during *E. granulosus* cyst development. (A) Box plot showing the comparative distribution of the weight (g) of cysts recovered from untreated (C) and Met-treated (Met, 50 mg/kg/d) mice. The treatment was initiated at the time of infection and followed during 60 days. At 4 months p.i., mice were necropsied. A significant cyst weight reduction (**p < 0.01) was achieved in treated animals. (B) Representative SEM (a, b, e, f) and TEM (c, d, g, h) images of hydatid cysts recovered from untreated control mice (a-d) compared with Met-treated mice (e-h). Bars indicate: 50 µmin (a, e), 10 µmin (b, f) and 1 µmin (c, d, g, h). II, laminated layer; mt, microtriches; dc, distal cytoplasm; gl, germinal layer; nu, nucleus; ly, lysosomes (arrowheads); ve: vesicles (double-headed arrow); gly, glycogen storage; a, autophagosomes; al, autophagolysosome; cc, calcareous corpuscles.

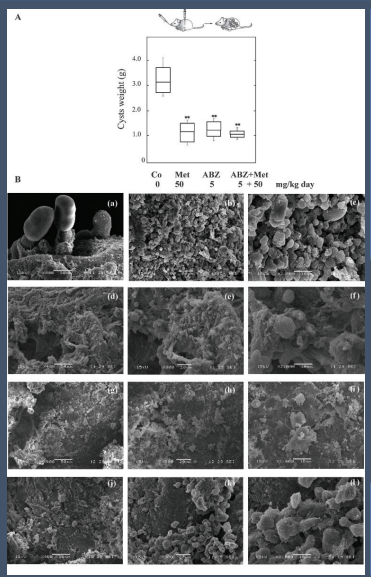


Fig 6. Chemoprophylactic activity of metformin during *E. multilocularis* cyst development. (A) Box plots showing the comparative distribution of the weight (g) of cysts recovered from untreated mice (C) and treated with albendazole (ABZ, 5 mg/kg/d), metformin (Met, 50 mg/kg/d) and the combination of both drugs (ABZ+Met) for 60 days from the time of infection. The weight of cysts was significantly decreased upon all treatments compared with the control (***$p < 0.05$). (B) Representative SEM images of cysts recovered from untreated mice (a, b, c) or treated with Met (d, e, f), ABZ (g, h, i) and ABZ+Met (j, k, l).

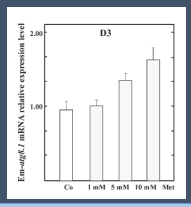
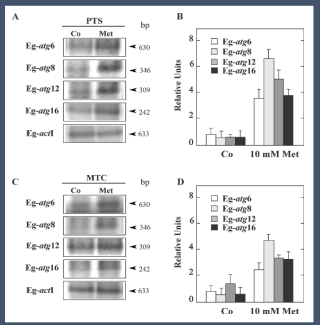


Fig 7. Transcriptional expression of Em-*atg8* in control and metformin-treated primary cells. Transcription levels of Em-*atg8* were increased after 3 days of incubation with 1, 5 and 10 mM Met.



GENE PROMOTER SEQUENCE upstream ATG

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Ce-sod3  GTGCGGGTGTTTACGCGTTTTCAA - 80   (Furuyama et al., 2000)
Hs-glnA  AGGCCTGTCCTTTACTCCTGAAAGA -2520  (van der Vos et al., 2012)
Eg-atg8  ATTACACTGTTTACAGTTGGAGC - 866   (Loos et al., 2014)
Eg-atg12 ATGATGATGTTTACATTGAGAACC -1031  (Loos et al., 2014)
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Fig.3. Transcriptional expression of *atg* genes in control and metformin-treated *E. granulosus* protoscoleces (PTS) and metacestodes (MTC). The expression was assessed by RT-PCR (a, c) and qPCR (b, d) analysis using total RNA extracted from control and Met-treated PTS (a, b) and MTC (c, d). Transcription levels of Eg-atg6-8-12-16 were increased in both larval stages after 2 days of incubation with 10 mM Met. (E) Sequence alignment of FoxO-activated promoters in metazoan (Ce-sod3, *C. elegans* superoxide dismutase 3 -PRJNA13758-; Hs-glnA, *H. sapiens* glutamine synthetase - NP_001028216- ; Eg-atg8 and Eg-atg12 reported in Loos *et al.*, 2014). The consensus for FoxO-binding site is boxed.

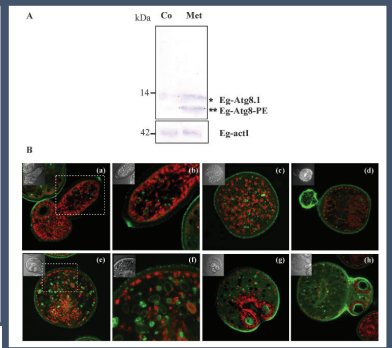

Fig.4. Detection and immunolocalization of Eg-Atg8 in control and metformin-treated protoscoleces. (A) Eg-Atg8/LC3 protein, which is considered the most reliable autophagy marker, was detected by immunoblotting in both soluble (Eg-Atg8.1) and membrane-bound (Eg-Atg8-PE) forms, from total protein extracts of control and Met-treated PTS. The expression was increased in the treated sample (10 mM Met – 2 days). (B) The protein was further detected by *in toto* assays in the tegument, the excretory system and the calcareous corpuscles of control (a-d) and treated (e-h) PTS.

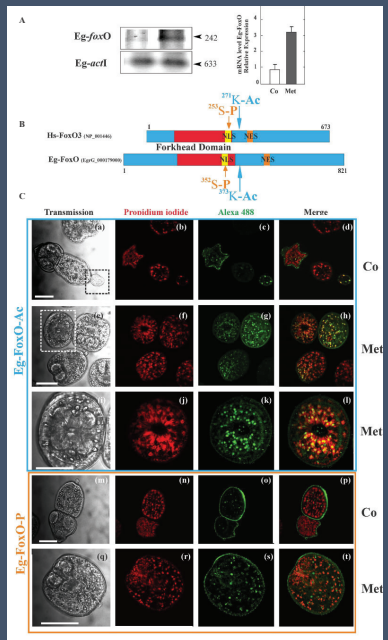

Fig.5. Transcriptional expression and immunolocalization of Eg-FoxO in control and metformin-treated protoscoleces. (A) Transcription level of Eg-*foxO* was increased in PTS after 2 days of incubation with 10 mM Met. (B) Schematic representation of *Homo sapiens* FoxO3 and of the only predicted FoxO protein from the *E. granulosus* genome. Identification of Forkhead domain (red), nuclear localization signal (NLS, yellow) and nuclear exportation signal (NES, orange). (C) Subcellular immunolocalization of an acetylated (Eg-FoxO-Ac) and a phosphorylated (Eg-FoxO-P) form of Eg-FoxO in control (Co) and treated (Met) PTS. Images of protoscoleces visualized by light transmitted microscopy (first column on the left) and by fluorescence confocal microscopy stained with propidium iodide -red fluorescence, second column on the left-, revealed with Ac-FoxO antibody conjugated with Alexa 488-green fluorescence, third column-, obtained by overlapping of the two fluorescence reactions (last column on the right). The punctuate staining for Eg-FoxO expression was evenly detected in both nucleus and cytoplasm. Nuclear expression is observed in yellow/orange, corresponding to the merged fluorescences.

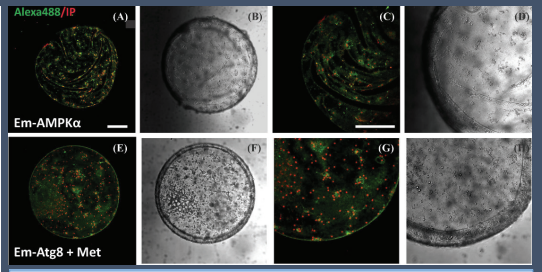

Fig 8. Em-AMPK and Em-Atg8 expression. By *in toto* immunolocalization assays, we detected the expression and subcellular localization of Em-AMPK (a-d) and Em-Atg8/LC3 (e-h) (both conjugated Alexa 488-green) in the germinal layer of *in vitro* obtained metacestodes. Bars 50 µm.

CONCLUSIONS

Oral administration of Met (50 mg/kg/day) in *Echinococcus granulosus* and *E. multilocularis*-infected mice was highly effective in reducing the weight of parasite cysts in chemotherapeutic protocols (Fig 2 and Fig. 6).

The energy stress induced by Metformin (Loos & Cumino, 2015) leads to autophagy activation in *Echinococcus* spp. (Fig. 3, 4 and 8).

Echinococcus autophagy could be regulated by transcription-dependent up-regulation via Eg-FoxO transcription factor (Fig 3 and Fig 5a-b)

Although, the phosphorylation of Eg-FoxO on Ser-352 (possibly by AKT, as described Calnan & Brunet, 2008), induces its non-functional-cytoplasmic localization, the acetylation on Lys-373 leads to its activation and nuclear localization (Fig. 5).